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(54) **MEANS AND METHODS FOR PRODUCING HIGH AFFINITY ANTIBODIES**

MITTEL UND METHODEN ZUR PRODUKTION VON HOCH AFFINEN ANTIKÖRPERN

MOYENS ET METHODES DE PRODUCTION D' ANTICORPS DE HAUTE AFFINITÉ

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Description

[0001] The invention relates to the field of cell biology. More specifically, the invention relates to the field of antibody production.

[0002] *Ex vivo* B-cell cultures are important tools in current biological and medical applications. One important application is culturing antibody producing cells in order to harvest antibodies, preferably monoclonal antibodies. Monoclonal antibodies (mAbs) represent multiple identical copies of a single antibody molecule. Amongst the benefits of mAbs is their specificity for the same epitope on an antigen. This specificity confers certain clinical advantages on mAbs over more conventional treatments while offering patients an effective, well tolerated therapy option with generally low side effects. Moreover mAbs are useful for biological and medical research.

[0003] Mature B-cells can be cultured *in vitro* under conditions which mimic some key aspects of the germinal centre (GC) reaction; that is, activation of B-cells with CD40 ligand (L) and the presence of cytokines like interleukin (IL)-4, IL-10 or IL-21. While B-cells cultured with CD40L, IL-2 and IL-4 produce very little Ig, addition of IL-21 leads to differentiation to plasma cells accompanied by high Ig secretion. Although this *in vitro* system has proven useful to study some aspects of B-cell differentiation, both naive IgD+ B-cells and switched IgD- memory B-cells eventually differentiate into terminally differentiated plasma cells, which is accompanied by cell cycle arrest precluding the generation of long-term antigen-specific BCR positive cell lines.

[0004] Recent advances have provided insight into how multiple transcription factors, including B-lymphocyte-induced maturation protein 1 (BLIMP1) and B-cell lymphoma (BCL)6 control development of GC B-cells into terminally arrested, antibody-producing plasma cells. The transcriptional repressor BCL6 has been shown to prevent plasma cell differentiation. BCL6 is highly expressed in GC B-cells were it facilitates expansion of B-cells by downregulating p53 and prevents premature differentiation of GC cells into plasma cells by negatively regulating BLIMP1.

[0005] An improved method for generating an antibody-producing plasmablast-like B-cell was recently described in WO 2007/067046. According to this method, the amount of BCL6 and a Bcl-2 family member, preferably Bcl-xL, are modulated in a B-cell, preferably a memory B-cell, to generate an antibody-producing plasmablast-like B-cell. In WO 2007/067046 the amount of BCL6 and/or Bcl-xL expression product is either directly or indirectly influenced. Preferably the amounts of both BCL6 and Bcl-xL expression products within said antibody producing cell are increased, since both expression products are involved in the stability of an antibody producing B-cell. Said Bcl-xL is a member of the anti-apoptotic Bcl-2 family. Processes that are controlled by the Bcl-2 family, which includes both pro- and anti-apoptotic proteins, relate to the mitochondrial pathway of apoptosis. This pathway proceeds when molecules sequestered between the outer and inner mitochondrial membranes are released into the cytosol by mitochondrial outer membrane permeabilization. The pro-apoptotic family members can be divided in two classes. The effector molecules Bax and Bak, which contain so-called Bcl-2 homology domain 3 (BH3) domains, are involved in permeablilizing the outer mitochondrial membrane by forming proteolipid pores; the pro-apoptotic BH3-only proteins (Bad, Bik, Bim, Bid, Hrk, Bmf, bNIP3, Puma and Noxa) function upon different cellular stresses by protein-protein interactions with other (anti-apoptotic) Bcl-2 family members.

[0006] Anti-apoptotic Bcl-2 family members Bcl-2, Bcl-xL, Bcl-w, A1 and Mcl-1 are generally integrated with the outer mitochondrial membrane. They directly bind and inhibit the pro-apoptotic Bcl-2 proteins to protect mitochondrial membrane integrity.

[0007] In such a method it is further preferred that said antibody producing plasmablast-like B-cell is incubated with IL 21 and CD40L. A B-cell, such as an antibody producing plasmablast-like B-cell, is preferably cultured in the presence of CD40L since replication of most B-cells is favoured by CD40L. It is furthermore preferred that STAT3 is activated in said antibody producing B-cell. Activation of STAT3 can be achieved in a variety of ways. Preferably, STAT3 is activated by providing an antibody producing cell with a cytokine. Cytokines, being naturally involved in B-cell differentiation, are very effective in regulating STAT proteins. Very effective activators of STAT3 are IL 2, IL 10, IL 21 and IL 6, but also IL 7, IL 9, IL 15, IL 23 and IL 27 are known to activate STAT3. Additionally, or alternatively, STAT3 activation is accomplished by transfer into a B-cell of a nucleic acid encoding a mutant of STAT3 that confers constitutive activation to STAT3. (Sean A Diehl, Heike Schmidlin, Maho Nagasawa, Simon D van Haren, Mark J Kwakkenbos, Etsuko Yasuda, Tim Beaumont, Ferenc A Scheeren, Hergen Spits STAT3-mediated up-regulation of BLIMP1 is coordinated with BCL6 down-regulation to control human plasma cell differentiation J Immunol 2008 vol. 180 (7) pp. 4805-15)

[0008] Most preferably IL 21 is used, since IL 21 is particularly suitable for influencing the stability of an antibody producing plasmablast-like B-cell. In addition to upregulating STAT3, IL 21 is capable of upregulating Blimp 1 expression even when Blimp 1 expression is counteracted by BCL6. With the methods disclosed in WO 2007/067046, it has become possible to increase the replicative life span of an antibody producing cell since it is possible to maintain a B-cell in a developmental stage wherein replication occurs. In earlier *ex vivo* B-cell cultures the replicative life span was only a few weeks to two months. During this time the cultured cells lose their capability of replicating and die. With a method as disclosed in WO 2007/067046, however, it has become possible to prolong the replicative life span of antibody producing memory B-cells, so that *ex vivo* cultures are generated comprising plasmablast-like B-cells that are capable of replicating

and producing antibody.

[0009] Although these methods enable the production of antibodies that efficiently target an antigen of interest, improvement of antibody characteristics, such as binding affinity, is often desired. Binding characteristics are therefore regularly altered by introducing mutations in the encoding nucleic acid, preferably in the CDR encoding region, and testing the resulting antibodies. This is, however, time consuming. Alternative methods for obtaining high affinity antibodies are therefore desired.

[0010] It is an object of the present invention to provide methods for producing and/or selecting high affinity antibodies.

[0011] The invention provides means and method for obtaining a B-cell population, starting from a given B-cell culture, which population has a higher average binding capacity than the original B-cell culture. Preferably, a monoclonal B-cell population is produced, starting from a monoclonal B-cell culture. The invention provides a simple and elegant way of obtaining B-cell populations with an increased average binding capacity, without the need for laborious mutation techniques.

[0012] The invention provides a method according to the claims for producing antibodies specific for an antigen of interest.

[0013] Within a population of monoclonal B-cells capable of producing antibody specific for an antigen of interest, it is possible to select, in step e) of a method according to the invention, at least one, optionally more than one, such as for instance 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 25 or 50 B-cells with a binding capacity for said antigen of interest that is higher than the average binding capacity of said population of B-cells for said antigen of interest. Such B-cells with a higher binding capacity for an antigen of interest than the average binding capacity of the population of B-cells for said antigen of interest are herein also called "high-affinity B-cells". One possible reason for a difference in binding capacity between multiple B-cells in a monoclonal population of B-cells is that the expression of the BCR varies between B-cells in said population. A B-cell with a relatively high expression of the BCR will bind more antigen of interest than a B-cell with a relatively low expression of the BCR. However, it is expected that antibodies produced by B-cells with different expression of the BCR, have the same binding affinity. The present inventors surprisingly found that, besides a relatively high BCR expression, a collection of high-affinity B-cells produce antibodies specific for the antigen of interest which bind said antigen with a higher affinity than the average affinity of antibodies produced by said population of B-cells. Even more surprisingly, the inventors found that the B cell cultures obtained with a method of the invention contained cells that bound antigen with a higher affinity than the average B cell in the original culture. Single B cells can thus be isolated from a given B cell population on the basis of their higher binding capacity by methods known in the art and be expanded to a new B cell population in at least three weeks. These new B cells produce antibodies that have a higher affinity than the antibodies produced by the original B cell population that the new B cells are derived from. This finding is contrary to expectations because a person skilled in the art would expect that after isolation of one B cell (subclone) from an already monoclonal population of said B-cells, the affinity for the antigen of antibody produced by the progeny of the subclone of said already monoclonal B cell population will return to the average affinity for the antigen, comparable to the average affinity of the population of B-cells from which the at least one B-cell was selected.

[0014] Thus, in one embodiment in step a) of a method according to the invention preferably a single B-cell is selected, for instance from a polyclonal population of B-cells. The single B-cell is subsequently expanded into a monoclonal population of B-cells in steps b) to d). This is for instance achieved using a method as described in WO 2007/067046, which is discussed herein before. Hence, in step d), a monoclonal B-cell line specific for an antigen of interest is obtained. In principle, all B-cells in the monoclonal B-cell line produce essentially the same antibodies specific for said antigen, although small differences in the affinity for said antigen may be present between cells of said monoclonal B-cell line, i.e. some B-cells in the monoclonal population produce antibodies with an affinity which is slightly higher than the average affinity and some B-cells in the monoclonal population produce antibodies with a slightly lower affinity. The population of B cells becomes slightly heterogeneous again. In step e), at least one of such B-cells with a higher affinity than the average affinity is selected from the monoclonal B-cell line. In step f) the B-cell or B-cells selected in step e) are subsequently cultured into a second, preferably monoclonal, B-cell line. The present invention provides the insight that this second, preferably monoclonal, B-cell line has an average affinity that is higher than the average affinity of the original monoclonal B-cell population obtained in step d). As described above, it was surprisingly found that the high affinity of a selected B-cell is maintained after culturing, even if culturing takes place during a prolonged period of time, instead of returning to the average affinity of the original population. Thus, the second monoclonal population of B-cells cultured in step f) has a higher average affinity for the antigen than the monoclonal population of B-cells cultured in step d). Similarly, the affinity of most B-cells in the second monoclonal population of step f) is higher than the affinity of most B-cells in a monoclonal population of step d).

[0015] Provided is a method according to the claims for producing antibodies specific for an antigen of interest. Antibodies are obtained which have an affinity for said antigen of interest which is higher than the average affinity for said antigen of interest of antibodies produced by B-cells of said first monoclonal B-cell line.

[0016] In another embodiment, more than one B-cell is selected in step a) of a method of the invention, for instance 2, 3, 4, 5, 10, 15, 25, 50 or 100 B-cells. The B-cells are for instance selected from a polyclonal population of B-cells or

from a biological sample. The selected B-cells are subsequently expended into a population of B-cells in steps b) to d), for instance using a method as described in WO 2007/067046. The obtained B-cell population is thus a (second) polyclonal B-cell population. Thereafter, and before step e) of a method of the invention is carried out, a monoclonal population of B-cells is preferably produced. This is for instance done by selecting a single B-cell from said (second) polyclonal population of B-cells using Fluorescence Activated Cell Sorting or limiting dilution, which are explained herein below, and expanding said selected single B-cell to a monoclonal population of B-cells. Then, step e) of a method of the invention is carried out, in which at least one B-cell with a higher affinity than the average affinity of the monoclonal B-cell population is selected. In step f) the B-cell or B-cells selected in step e) are subsequently cultured into a second monoclonal B-cell line, after which antibodies produced by said second monoclonal B-cell line are obtained in step g).

[0017] A method as described herein allows for obtaining improved, high affinity antibodies, preferably monoclonal antibodies, without the use of recombinant techniques. Before the present invention, affinity of (monoclonal) antibodies is increased using such recombinant techniques. The sequence of the nucleic acid encoding the antibody first needs to be determined. Subsequently one or more mutations are introduced into the sequence of the nucleic acid encoding the antibody. Then, the genes containing one or more mutations need to be expressed in a cell followed by production of antibodies in producer cells. Finally, the mutated antibody has to be tested for its binding capacity to the antigen of interest in order to determine whether antibody with an improved affinity for said antigen as compared with the non-mutated antibody is obtained. Such a process for improving the affinity of an antibody is elaborate and time-consuming. A method according to the present invention allows the production of high affinity antibody in a straight-forward and less elaborate process without the need of molecular engineering.

[0018] In one embodiment of the invention, after the step of selecting at least one high-affinity B-cell from said already monoclonal population of B-cells (step e) of a method of the invention as described above), said at least one high-affinity B-cell is allowed to expand into a population of B-cells, preferably a monoclonal B-cell line, again, after which another step of selecting at least one high-affinity B-cell from said new population of B-cells, preferably from said new monoclonal B-cell line, is performed. By repeating the steps of allowing expansion of a selected B-cell into a population and selecting at least one B-cell on the basis of its binding capacity for an antigen, i.e. repeating steps d) and e), it is possible to generate high affinity antibody producing B-cells. Preferably, by repeating the steps of expansion and selection as described above, it is possible to increase with each selection cycle the affinity of antibody produced by the resulting B-cell population for the antigen of interest.

[0019] A method is thus provided comprising, following step e) of a method of the invention, repeating the step of allowing expansion of at least one selected high-affinity B-cell into a population of B-cells, preferably a monoclonal B-cell line, and selecting again at least one high-affinity B-cell, i.e. repeating steps d) and e) of a method of the invention at least once. Said steps are for instance repeated once, but preferably twice, three times, four times, five times or even more times.

[0020] In one embodiment a method of the invention is provided wherein said at least one B-cell selected in step e) is cultured for at least four weeks. Preferably said at least one B-cell selected in step e) is cultured for at least six weeks, more preferably for at least nine weeks, more preferably for at least three months, more preferably for at least six months.

[0021] Without being bound to any theory, it is believed that differences in the affinity of antibodies for an antigen of interest within a population of monoclonal B-cells may result from processes mediated by Activation Induced Cytidine Deaminase (AID). Antigen-activated naive and memory B-cells in the germinal centre undergo extensive proliferation, accompanied by somatic hypermutations (SHM) and class-switch recombination (CSR) of Ig genes mediated by AID. AID deaminates deoxycytidine residues in immunoglobulin genes, which triggers antibody diversification. It was demonstrated in patent application US2008305076 that IL 21 induces BLIMP, BCL6 and AID expression, but does not directly induce somatic hypermutation. However, the present inventors found that AID is expressed in B-cells which are cultured according to a method as herein described. The expression of AID in (a B-cell which will develop into) an antibody producing B-cell allows the generation of novel immunoglobulins that harbor mutations that were not present in the original B-cell before transduction with BCL6 and an anti-apoptotic nucleic acid. Thus, culturing B-cells in which somatic hyper mutation is induced by expression of AID allows the generation of immunoglobulin variants which, for example, have a higher or lower affinity for an antigen of interest, or that are more stable, for example, in an aqueous solution or under increased salt conditions, or any combination thereof.

[0022] Upon selection of at least one high-affinity B-cell from said population of B-cells, AID is still expressed within said selected at least one B-cell. Therefore, after selection of such a B-cell, AID in said B-cell still allows the introduction of mutations in the immunoglobulin gene of the progeny of said B-cell. Somatic hypermutations in immunoglobuline genes occur preferentially in the CDR3 region of the Ig genes. Mutations introduced in the CDR3 region of the immunoglobulin are more likely to result in a reduced or lost binding affinity for an antigen of said immunoglobulin than in an increased binding affinity. The present inventors, however, did find increased binding affinity.

[0023] As used herein, the term "anti-apoptotic nucleic acid" refers to a nucleic acid which is capable of delaying and/or preventing apoptosis in a B-cell. Said anti-apoptotic nucleic acid is capable of delaying and/or preventing apoptosis in an antibody producing B-cell. An anti-apoptotic nucleic acid is used which comprises an exogenous nucleic acid. This

means that either a nucleic acid sequence is used which is not naturally expressed in B-cells, or that an additional copy of a naturally occurring nucleic acid is used, so that expression in the resulting B-cells is enhanced as compared to natural B-cells. Various anti-apoptotic nucleic acids are known in the art. A nucleic acid is used which is an anti-apoptotic member of the Bcl-2 family because anti-apoptotic Bcl-2 proteins are good apoptosis inhibiters. Many processes that are controlled by the Bcl-2 family (which family includes both pro- and anti-apoptotic proteins) relate to the mitochondrial pathway of apoptosis, as outlined in more detail herein below. Anti-apoptotic Bcl-2 family members Bcl-2, Bcl-xL, Bcl-w, A1 and Mcl-1 are generally integrated with the outer mitochondrial membrane. They directly bind and inhibit the pro-apoptotic proteins that belong to the Bcl-2 family to protect mitochondrial membrane integrity.

[0024] In a method according to the invention said anti-apoptotic nucleic acid encodes Bcl-xL. A combination of BCL6 and Bcl-xL nucleic acids is particularly suitable for immortalizing B-cells and long term culture of the resulting plasmablast-like B-cells. A combination of BCL6 and Bcl-xL stabilizes B-cells particularly well.

[0025] A population of B-cells according to the invention preferably is a monoclonal population of B-cells. An example of a population of B-cells according to the invention is a cell line of B-cells, preferably monoclonal B-cells. Hence, a population of B-cells according to the invention is most preferably a monoclonal B-cell line. Allowing expansion of said B-cell into a population of said B-cells is for instance accomplished by culturing said B-cell until a population of said B-cells is obtained.

[0026] Within a population of B-cells, even in a population of monoclonal B-cells, the binding capacity of the BCR's of the B-cells of said population, and the binding capacity of the antibodies produced by the B-cells of said population, is not equal. Instead, variation in said binding capacity exists. The average binding capacity of a population of B-cells is herein defined as the average of the binding capacity or average affinity of the BCR and/or antibody of all individual B-cells in said population. The average affinity for an antigen of interest of an antibody produced by a B-cell or by a population of B-cells is herein defined as the average of the affinities for said antigen of interest of the antibodies produced by all individual B-cells in said population. A high-affinity B-cell from a population of B-cells, preferably from a monoclonal B-cell line, according to the invention is preferably selected from the upper 40% of the B-cells of a population, preferably of a monoclonal B-cell line, with respect to binding capacity and/or affinity, preferably from the upper 30% of the B-cells of said population or monoclonal B-cell line, more preferably from the upper 25% of the B-cells of said population or monoclonal B-cell line, more preferably from the upper 20% of the B-cells of said population or monoclonal B-cell line, more preferably from the upper 15% of the B-cells of said population or monoclonal B-cell line, more preferably from the upper 10% of the B-cells of said population or monoclonal B-cell line, more preferably from the upper 1% of the B-cells of said population or monoclonal B-cell line. In one embodiment, one high-affinity B-cell is selected from the upper 1% of the B-cells of a population or monoclonal B-cell line with respect to binding capacity and/or affinity.

[0027] The average affinity for an antigen of interest of antibody produced by a population of B-cells, preferably by a monoclonal B-cell line, cultured from at least one high-affinity B-cell according to the invention is preferably at least 1.1 times the average affinity for said antigen of interest of the population of B-cells from which the at least one high-affinity B-cell was selected, more preferably at least 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 3.0, 3.5, 4.0, 5.0, 10.0, 20, 50, 100 times, or more, the average affinity for said antigen of interest.

[0028] The affinity of an antibody can be determined using any method known to a person skilled in the art. The affinity of an antibody is for instance determined using Enzyme-linked immunosorbent assay (ELISA), Surface Plasmon Resonance (such as Biacore) or Octet (ForteBio). Surface Plasmon Resonance (SPR) and Octet are techniques to measure biomolecular interactions in real-time in a label free environment. For SPR, one of the interactants, for instance an antibody, is immobilized to the sensor surface, the other, for instance antigen, is free in solution and passed over the surface. Association and dissociation is measured in arbitrary units and preferably displayed in a sensorgram. Any change in the number of molecules bound to the biosensor tip causes a shift in the interference pattern that can be measured in real-time. Using Octet the interference pattern of white light reflected from two surfaces, a layer of immobilized protein on the biosensor tip, and an internal reference layer is analyzed. The binding between a ligand immobilized on the biosensor tip surface, for instance an antibody, and a protein in solution, for instance an antigen of interest, produces an increase in optical thickness at the biosensor tip, which results in a wavelength shift which is a direct measure of the change in thickness of the biological layer. ELISA comprises immobilizing a protein, for instance the antigen of interest, on the surface of the solid support, for example a 96-well plate, and applying a sample to be detected or quantified on the solid support. Alternatively, a capture antibody is fixated on the surface of a solid support after which a sample containing the protein to be detected or quantified is applied to the immobilized capture antibody allowing the protein of interest to bind. Non-binding proteins are than washed away. Subsequently a specific antibody conjugated to a label or an enzyme (or a primary antibody followed by a secondary antibody conjugated to a label or an enzyme) is added to the solid support. Preferably the affinity constant (K_D) of an antibody produced by a B-cell according to the invention is determined.

[0029] Binding of a B-cell according to the invention to an antigen of interest can be measured using any method known to a person skilled in the art. For instance, an antigen of interest is labelled with, for example, a fluorescent label. Detection of binding can subsequently be determined by various techniques, among which fluoresce microscopy and

Fluorescence Activated Cell Sorting (FACS). FACS allows separation of cells in a suspension on the basis of size and the fluorescence of conjugated antibodies directed against surface antigens.

[0030] Selecting at least one high-affinity B-cell from a population of B-cells, preferably from a monoclonal B-cell line, can be performed using any method known to a person skilled in the art. Selection of at least one high-affinity B-cell according to the invention is for instance performed by cell sorting for instance using FACS (see above) or limited dilution. Limited dilution comprises the serial dilution of a suspension of cells, for instance B-cells, until a single cell is present in a given volume. Subsequently, the binding capacity of each B-cell (after expansion of single cells to a population) is tested to allow selection of a B-cell producing antibodies with a high affinity for antigen.

[0031] A B-cell capable of producing antibody is defined as a B-cell which B-cell is capable of producing and/or secreting antibody or a functional part thereof, and/or which cell is capable of developing into a cell which is capable of producing and/or secreting antibody or a functional part thereof.

[0032] A functional part of an antibody is defined as a part which has at least one same property as said antibody in kind, not necessarily in amount. Said functional part is preferably capable of binding a same antigen as said antibody, albeit not necessarily to the same extent. A functional part of an antibody preferably comprises a single domain antibody, a single chain antibody, a FAB fragment, a nanobody, an unibody, a single chain variable fragment (scFv), or a $F(ab')_2$ fragment.

[0033] Non-limiting examples of a B-cell used or selected in a method according to the invention include B-cells derived from a human individual, rodent, rabbit, llama, pig, cow, goat, horse, ape, chimpanzee, macaque and gorilla. Preferably, said B-cell is a human cell, a murine cell, a rabbit cell, an ape cell, a chimpanzee cell, a macaque cell and/or a llama cell. Most preferably, said B-cell is a human B-cell.

[0034] In a preferred embodiment, a memory B-cell is selected in step a) of the method as described herein, for instance a human memory B-cell. In a particularly preferred embodiment, said memory B-cell is a peripheral blood memory B-cell. Peripheral blood memory B-cells are easily obtained, without much discomfort for the individual from which they are derived, and appear to be very suitable for use in a method according to the present invention.

[0035] A B-cell or a population of B-cells, preferably a monoclonal B-cell line, obtained with a method according to the invention is preferably stable for at least four weeks, more preferably at least six weeks, more preferably at least nine weeks, more preferably for at least three months, more preferably for at least six months, meaning that such B-cells are capable of both replicating and producing antibody, or capable of replicating and developing into a cell that produces antibody, during said time periods. B-cells obtained by a method according to the invention preferably comprise cells producing IgM or cells producing other immunoglobulin isotypes like IgG, or IgA, or IgE, preferably IgG. A B-cell obtained by a method according to the invention is particularly suitable for use in producing an antibody producing cell line. High-affinity B-cells or a population or monoclonal B-cell line of high-affinity B-cells obtained by a method according to the invention are preferably cultured *ex vivo* and antibody is preferably collected for further use. Antibodies or functional parts thereof produced with a method according to the invention are useful for a wide variety of applications, such as for instance therapeutic, prophylactic and diagnostic applications, as well as research purposes and *ex vivo* experiments. For instance, a screening assay is performed wherein antibodies or functional parts obtained by a method according to the invention are incubated with a sample in order to determine whether an antigen of interest is present.

[0036] In one embodiment, a high-affinity B-cell or a population or monoclonal B-cell line of high-affinity B-cells obtained by a method according to the invention comprises a human B-cell, capable of producing human antibody, because human antibodies are particularly suitable for therapeutic and/or prophylactic applications in human individuals.

[0037] The expression of BCL6 in a B-cell is induced, enhanced and/or maintained in a variety of ways. In a method according to the invention a B-cell is provided with a nucleic acid encoding BCL6.

[0038] In a method according to the invention said B-cells are at least at some stage incubated with IL 21 and CD40L. A B-cell, such as an antibody producing plasmablast-like B-cell, is preferably cultured in the presence of CD40L since replication of most B-cells is favored by CD40L. It is furthermore preferred that STAT3 is activated in said B-cell. Most preferably IL 21 is used for upregulating STAT3, since IL 21 is particularly suitable for influencing the stability of a B-cell according to the invention. In addition to upregulating STAT3, IL 21 is capable of upregulating Blimp 1 expression even when Blimp 1 expression is counteracted by BCL6.

[0039] In another embodiment the amount of Blimp-1 expression product in said B-cell selected in step a) of a method according to the invention is directly or indirectly controlled. The amount of Blimp-1 expression product can be controlled in various ways, for instance by regulating STAT3 or a functional part, derivative or analogue thereof. STAT3 is activated in a variety of ways. Preferably, STAT3 is activated by providing a B-cell according to the invention with a cytokine. Cytokines, being naturally involved in B cell differentiation, are very effective in regulating STAT proteins. Very effective activators of STAT3 are IL-21 and IL-6, but also IL-2, IL-7, IL-10, IL-15 and IL-27 are known to activate STAT3. Moreover, Toll-like receptors (TLRs) which are involved in innate immunity are also capable of activating STAT3. Most preferably IL-21 is used. IL-21 is capable of upregulating Blimp-1 expression even when Blimp-1 expression is counteracted by BCL6.

[0040] By a functional part of STAT3 is meant a proteinaceous molecule that has the same capability - in kind, not necessarily in amount - of influencing the stability of an antibody producing cell as compared to STAT3. A functional

part of a STAT3 protein is for instance devoid of amino acids that are not, or only very little, involved in said capability. A derivative of STAT3 is defined as a protein which has been altered such that the capability of said protein of influencing the stability of an antibody producing cell is essentially the same in kind, not necessarily in amount. A derivative is provided in many ways, for instance through conservative amino acid substitution wherein one amino acid is substituted by another amino acid with generally similar properties (size, hydrophobicity, etc), such that the overall functioning is likely not to be seriously affected. A derivative for instance comprises a fusion protein whose activity depends on the presence of 4 hydroxy-tamoxifen (4HT). An analogue of STAT3 is defined as a molecule having the same capability of influencing the stability of an antibody producing cell in kind, not necessarily in amount. Said analogue is not necessarily derived from said STAT3 protein.

[0041] A method according to the invention is preferably used for generating a cell line of high-affinity B-cells that is stable for at least one week, preferably at least one month, more preferably at least three months, more preferably at least six months so that commercial high-affinity antibody production has become possible. Preferably a stable cell line capable of producing monoclonal high-affinity antibodies is produced. This is preferably performed by using memory B-cells that have for instance been isolated from a sample by selection for CD19 (B-cell marker) and cell surface IgG and/or CD27 (to mark memory cells). Furthermore, a memory B-cell capable of specifically binding an antigen of interest is for instance selected in a binding assay using said antigen of interest. Subsequently, BCL6 and Bcl-XL are co-expressed in said B-cell, resulting in a population of cells specific for said antigen of interest. Preferably only one memory cell is selected in step a) of a method as described herein, so that a B-cell population according to the invention producing monoclonal antibodies (a monoclonal B-cell line) is obtained.

[0042] In one embodiment, a B-cell, preferably but not necessarily a memory B-cell, that originates from an individual which had been previously exposed to an antigen of interest, is used in a method according to the invention. However, this is not necessary. It is also possible to use a B-cell from an individual that has not been exposed to said antigen of interest. For instance, a B-cell is used that is specific for another antigen but shows cross-reactivity with the antigen of interest. As another example, a B-cell is used that is selected from a naive B-cell population of an individual. The naive B-cell population of an individual may contain B-cells that show reactivity with an antigen of interest even though the individual has not been exposed to said antigen of interest. Such B-cell from a naive B-cell population is for instance selected using labelled antigen of interest.

[0043] The invention furthermore describes isolated or recombinant B-cells and populations of B-cells, preferably monoclonal B-cell lines, obtained by a method according to the invention. Such high-affinity B-cells are preferably stable for at least one week, preferably for at least one month, more preferably for at least three months, more preferably for at least six months, meaning that the B-cell is capable of both replicating and producing antibody, or capable of replicating and developing into a cell that produces antibody, during said time periods. B-cells obtained by a method according to the invention preferably comprise cells producing IgM or cells producing other immunoglobulin isotypes like IgG, or IgA, or IgE, preferably IgG. A B-cell obtained by a method according to the invention is particularly suitable for use in producing an antibody producing cell line. High-affinity B-cells obtained by a method according to the invention are preferably cultured *ex vivo* and antibody is preferably collected for further use. Antibodies obtained from a B-cell or from a B-cell population or monoclonal cell line obtained by a method according to the invention are also described. High-affinity antibodies or functional parts thereof produced with a method according to the invention are useful for a wide variety of applications, such as for instance therapeutic, prophylactic and diagnostic applications, as well as research purposes and *ex vivo* experiments. For instance, a screening assay is performed wherein antibodies or functional parts obtained by a method according to the invention are incubated with a sample in order to determine whether an antigen of interest is present.

[0044] B-cells generated with a method according to the invention are particularly suitable for producing high-affinity antibodies against an antigen of interest. In one preferred embodiment, however, the genes encoding the Ig heavy and/or light chains are isolated from said cell and expressed in a second cell, such as for instance cells of a Chinese hamster ovary (CHO) cell line. Said second cell, also called herein a producer cell, is preferably adapted to commercial antibody production. Proliferation of said producer cell results in a producer cell line capable of producing antibody. Preferably, said producer cell line is suitable for producing compounds for use in humans. Hence, said producer cell line is preferably free of pathogenic agents such as pathogenic micro-organisms.

[0045] The invention is further explained by the following, non-limiting, examples.

Figure legends

[0046]

Figure 1. (A) Binding of labeled HA H3 protein to the BCR of H3 specific cells within a polyclonal B cell population. B cells that bind the H3 protein with high affinity were cloned by single cell sorting. After 2-3 weeks of culture the culture supernatant was screened for H3 specific antibodies. (B) Example of the screening performed to select H3

specific clones. (left panel) Screening by ELISA. Recombinant H3 protein was coated onto a plate followed by incubation with culture supernatant. Antibody binding was detected using anti-human-IgG-HRP. (right panel) Screening on cell surface expressed HA. H3N2 infected cells were incubated with B cell culture supernatant. Antibody binding was detected with a PE labelled goat anti-human F(ab') 2.

Figure 2. (Left) mRNA levels of AICDA in CD19+CD38+CD20+IgD- tonsillar GC B cells and CD19+IgG +CD27+ peripheral blood memory B cells compared to 23 BCL6- and Bcl-xL-transduced monoclonal cell lines. (Right) Selection of high or low binding subclones within an H3 specific clone. Boxed populations were selected by cell sorting and further expanded.

Figure 3. A) FACS analysis for the binding of labelled HA H3 to selected cells 13 days after their selection for higher or lower H3 BCR binding from a clonal cell. Increased or lowered H3 binding is maintained and stable after subcloning. B) FACS staining for the BCR of the different selected subpopulations. Increased or lowered levels of H3 binding to selected populations correlates with the BCR expression on the cell surface of these populations. Light grey line: B cells selected for high H3 binding; filled graph: B cells not selected (parental cells); dark grey line: B cells selected for low H3 binding.

Figure 4. H3 ELISA of the culture supernatant of the different (sub)populations. Secreted IgG from cells that were selected for higher binding to H3-APC protein show increased binding in the H3 ELISA compared to IgG from the non-sorted parental line. Top line: B cells selected for high H3 binding; middle line: B cells not selected (parental cells); bottom line: B cells selected for low H3 binding.

Figure 5. Selection of high or low affinity subclones within an H3 specific clone (AT10_004). Cells were stained with Alexa-647 labeled HA H3 antigen together with IgG-PE antibody. Circled populations were selected by cell sorting and further expanded.

Figure 6. FACS analysis for the binding of labeled HA H3 together with a BCR stain (either for the heavy chain, IgG-PE, or for the light chain, Kappa-PE) to selected cells 2 weeks after the third selection round for higher or lower H3 BCR binding and to the parental AT10_004.

Figure 7. Overview of the amino acid changes that were found in the selected subpopulations with increased or decreased affinity. Mutations in the sequence of AT10_004 that were associated with increased H3 antigen binding were incorporated in the AT10_004 sequence and these antibodies were produced recombinant in 239T cells and purified for further analysis (AT10_004 mutant B and AT10_004 mutant C).

Figure 8. SPR analysis of the binding of AT10_004 antibodies to HA H3. Association curves of antibodies AT10_004, AT10_004 mutant A, AT10_004 mutant B and AT10_004 mutant C.

Figure 9. Mean fluorescent intensity (MFI) of AT10_004 antibody variants binding to H3N2 infected cells in a FACS assay. Different concentrations of recombinant AT10_004, AT10_004 mutant B, AT10_004 mutant C and Rituximab (negative control) were incubated with H3N2 infected cells. Antibody binding was detected with PE labeled goat anti-human F(ab') 2. Plotted is the mean and the SEM of the MFI of the resulting PE signal.

Examples

Example 1

Generation of an anti-influenza hemaglutinin (HA) H3 specific monoclonal human antibody.

[0047] Human memory B cells were immortalized using the BCL6 / Bcl-xL technology described by Kwakkenbos et al. (Generation of stable monoclonal antibody-producing B cell receptor- positive human memory B cells by genetic programming. Nature Medicine (2010) vol. 16 (1) pp. 123-8 and patent application MEANS AND METHODS FOR INFLUENCING THE STABILITY OF ANTIBODY PRODUCING CELLS [WO 2007/067046]). In brief, BCL6 and Bcl-xL transduced cells (GFP positive) were cultured with CD40Ligand expressing L-cells and interleukin (IL)-21 before the HA H3 binding cells were sorted using the Fluorescence activated cell sorter (FACS)(Figure 1A). The Influenza HA protein (Protein Sciences) was labelled with Alexa Fluor 647 (Molecular Probes) and incubated with polyclonal cultured B cells. HA positive cells were sorted single cell per well and maintained in culture for 2 to 3 weeks before the clones were screened for HA binding by 1) ELISA or 2) binding to H3 infected cells (Figure 1B).

Example 2

Selection of a higher and lower affinity B cell clone.

[0048] Since the BCL6 Bcl-XL transduced B cells express the enzyme Activation Induced Deaminase (AID, gene nomenclature is AICDA) as described by Kwakkenbos et al. (Figure 2 left panel and 'Generation of stable monoclonal antibody-producing B cell receptor-positive human memory B cells by genetic programming' Nature Medicine (2010) vol. 16 (1) pp. 123-8) an individual B cell can make nucleotide changes in the immunoglobulin heavy and light chain. These changes may influence the binding affinity of the clones to its antigen. To determine if subclones of the HA H3 binding clone indeed can have a different binding profile, the H3 specific clone was again incubated with labelled HA H3 antigen. Using the FACS, a population of high HA H3 binding cells and a population of low HA H3 binding cells were sorted (Figure 2, right panel) and maintained in culture for at least 13 days before the B cell supernatant was harvested and tested.

Example 3

HA H3 high and low affinity sorted B cells express a stable but variable level of surface immunoglobulin

[0049] First we characterized the stability of the sorted cells by analyzing the binding capacity of the B cell receptor (BCR) to labelled HA H3 by FACS (Figure 3A). Since the HA H3 high sorted cells still showed higher binding abilities we next determined the surface immunoglobulin expression level by FACS (Figure 3B). It was observed that the cells sorted for a relative low binding capacity to HA H3 did express less immunoglobulin protein on the surface compared to cells sorted for high HA binding. This higher or lower BCR expression and BCR binding to HA H3 protein was maintained over time and became even more pronounced after a second round of sorting (data not shown).

Example 4

Affinity for HA H3 of the antibodies derived from the original and high and low affinity HA H3 binding cells

[0050] To determine the binding affinity of the antibodies produced by the different HA H3 recognizing B cells, the culture supernatant of the day 13 cultures of the original HA H3 binding cells and of the high and low affinity HA H3 binding cells was analyzed by ELISA. HA H3 (1ug/ml, Protein Sciences) was coated directly on the plate before the wells were incubated with the different B cell supernatants. Binding of the human IgGs to the HA H3 protein was detected with an anti-human polyclonal goat antibody that was HRP labelled. Secreted IgG from cells that were selected for higher binding to H3-APC protein show increased binding in the H3 ELISA compared to IgG from the non-sorted parental line (Figure 4).

Example 5

Combined BCR - antigen stain for the selection of high and low affinity clones.

[0051] In example 2 and 3 it is shown that selection of B cells, within the heterogeneous subpopulation of a monoclonal B cell clone, with the highest level of H3 binding may select for cells that have elevated levels of BCR expression. Thus when selection is done solely based on the level of H3 binding, cells that have increased antigen affinity but low levels of BCR expression might be excluded. To exclude the influence of the level of immunoglobulin expression on the selection of high affinity clones, a new set of selection rounds were performed using a combination of antigen staining (H3-Alexa-647) and BCR staining (Figure 5). BCR staining was performed with antibodies that bind to the heavy- or the light chain of the BCR. High H3 staining and low BCR staining indicates high antigen affinity, whereas low H3 staining and high BCR staining indicates low antigen affinity.

[0052] An HA H3 specific B cell clone (AT10_004) was cultured for 2-3 weeks to produce millions of cells before an antigen-BCR staining was performed. Cells that showed deviating antigen affinity, both positive and negative, were selected and sorted on a cell sorter. After 3 rounds of sorting and growing, FACS analysis was performed on these cells to determine differences in antigen binding. Cells that were sorted three times for increased- or decreased antigen binding show a clear shift in antigen binding compared to non-selected cells (Figure 6). Figure 6 demonstrates that increased or lowered H3 binding is maintained and stable after selection.

Example 6

Sequencing of the BCR from selected cells

[0053] We isolated total RNA with the RNeasy® mini kit (Qiagen) from AT10_004 and AT10_004 mutant B cell cultures selected for high or low affinity, generated cDNA from the RNA, performed PCR and analyzed the sequence of the heavy chain and light chain of the BCR. A mutation leading to an amino acid change at position 38 (CDR1) resulting in the exchange of the Glycine to an Alanine in the heavy chain was found for the cells that were sorted for decreased affinity (hereafter named mutant A). Mutations leading to amino acid changes in the light chain (compared to the parental AT10_004 sequence) were found for the increased affinity sorted cells. Sequence analysis showed a change of amino acid 108 (CDR3) in the kappa light chain from a Serine to a Tyrosine (hereafter named mutant B). An additional mutation at position 38 leading to replacement of Tyrosine to a Phenylalanine was found in some sequences (hereafter named mutant C) (Figure 7 and table 1). To produce recombinant AT10_004 and increased affinity mutants B and C mAb, we cloned the heavy and light variable regions in frame with human IgG1 and Kappa constant regions into a pcDNA3.1 (Invitrogen) based vector and transiently transfected 293T cells. We purified recombinant mAb from the culture supernatant with an ÄKTA (GE healthcare).

Example 7

Surface plasmon resonance (SPR) analysis

[0054] SPR analysis was performed on an IBIS MX96 SPR imaging system (IBIS Technologies BV., Enschede, The Netherlands) as described (Lokate et al., 2007, J. Am. Chem. Soc. 129:14013-140318). In short, one SPR analysis cycle consists of one or more incubation steps, in which analytes are flushed over a coated sensor, followed by a regeneration step, in which any bound analyte is removed from the sensor. Multiple cycles can be performed in one experiment. Dilution series (concentration ranging from 0.30 to 10 μg/ml) of AT10_004 and AT10_004 mutant antibody in coupling buffer (PBS + 0.03 % Tween20 + 0.01 mg/ml BSA) were immobilized during 99 minutes on an human-IgG-specific gold-film gel-type SPR-chip (Ssens, Enschede, The Netherlands) using a continuous flow microspotter device (Wasatch Microfluidics, Salt Lake City, UT, USA). After spotting, the sensor was washed three times with PBS + 0.03 % Tween20 (PBST).

[0055] To block any unoccupied sites in the anti-IgG coated SPR chip, the chip was first injected with a non-specific human IgG (rituximab, 10 μg/ml in PBST) and incubated for 45 minutes, followed by 100 minutes incubation with PBST. After this blocking step, two blank injections cycles were done, each consisting of 45 minutes injection with empty assay buffer (1x PBST + 0.01 % BSA) followed by 100 minutes incubation with PBST. Then, the sensor was injected with 1 μg/ml recombinant influenza HA3-protein (from H3N2, Wyoming, 03/2003, Sino Biological inc., Beijing, P.R. China) in assay buffer and incubated for 45 minutes. Subsequently, the sensor is washed with PBST and incubated for 100 minutes (to measure complex dissociation). Obtained data was analyzed using Sprint software (version 1.6.8.0, IBIS Technologies BV., Enschede, The Netherlands). Binding constants were fitted using Scrubber2 software (Biologic Software, Campbell, Australia). Figure 8 shows that recombinant HA3 does not associate with AT10_004 mutant A. An increased association rate of HA3 for AT10_004 mutant B and C is seen compared to the non-mutated AT10_004. The binding constants obtained for AT10_004 and each mutant are shown in table 2.

Example 8

Antibody binding to virus infected cells

[0056] To test the binding capacity of AT10_004 and the AT10_004 mutants to virus infected cells we performed FACS analysis on Influenza H3N2 (A/Netherlands/177/2008) infected cells. MDCK-SIAT cells were grown in a T175 culture flask to 80-100% confluency in DMEM/FCS/PS/G418. The cell layer was washed 2x with 10 ml PBS after which 15 ml of Optimem/PS/G418/Trypsin was added. Subsequently 0.5 ml of 100.000 TCID50 Influenza virus was added to the flask and cells were cultured at 37°C. After 24-48 hr the cells were washed 2x with 10 ml PBS and detached from the plastic using Trypsin-EDTA. Cells were counted and frozen at -150°C until use. The infected cells were defrosted and incubated with AT10_004 (mutant) antibodies or Rituximab (as negative control) at several concentrations for 30 minutes at 4°C and then washed 2x with 150 μl PBS/2%FCS. Antibody binding was detected with anti-human IgG-PE (Southern Biotech) and analyzed on a Guava easyCyte 8HT, Millipore). AT10_004 mutants B and C both show increased staining intensity on H3N2 infected cells compared to the parental AT10_004 antibody (Figure 9).

Table 1. Amino acid and nucleotide sequences of antibodies AT10-004 and AT10-004 mutants A, B and C. In the mutant sequences mutations as compared to antibody AT10-004 are indicated in bold and underlined

SEQ ID NO	Antibody	Identity	Sequence
1	AT10_004	Heavy chain CDR1	RHGIS
2	AT10_004	Heavy chain CDR2	WISAYTGDTDYAQKFQG
3	AT10_004	Heavy chain CDR3	LRLQGEVVVPPSQSNWFDP
4	AT10_004	Light chain CDR1	RASQSVSRYLA
5	AT10_004	Light chain CDR2	DASNRAT
6	AT10_004	Light chain CDR3	QQRSNWLK
7	AT10_004	Heavy chain	QVQLVQSGAEVRKPGASVKVSCKASGYTFTRHGISWVRQAPGQGLEWMGWISAYTGDTDY AQKFQGRVTMTTDTSTNTAYMELRSLRSDDAAVYYCARLRLQGEVVVPPSQSNWFDPWGQ GTLVTVSS
8	AT10_004	Light chain	EIVLTQSPATLSLYPGERATLSCRASQSVSRYLAWYQQKPGQAPRLLIYDASNRATGIPARFS GSGSGTDFTLTISSLEPEDFAVYYCQQRSNWLKITFGQGTRLEIKGTV
9	AT10_004	Heavy chain CDR1	agg cat ggt atc agc
10	AT10_004	Heavy chain CDR2	tgg atc agc gct tac act ggt gac aca gac tat gca cag aaa ttc cag ggg
11	AT10_004	Heavy chain CDR3	ctt cgt ttg cag ggt gaa gtg gtg gtc cct cct agt caa tcc aat tgg ttc gac ccc
12	AT10_004	Light chain CDR1	agg gcc agt cag agt gtt agc agg tac tta gcc
13	AT10_004	Light chain CDR2	gat gca tcc aac agg gcc act

(continued)

SEQ ID NO	Antibody	Identity	Sequence
14	AT10_004	Light chain CDR3	cag cag cgt gac aac tgg ctt aag
15	AT10_004	Heavy chain	cag gtt cag ctg gtg cag tct gga gct gag gtg agg aag cct ggg gcc tca gtg aag gtc tcc tgc aag gct tcc ggt tac acg ttt acc agg cat ggt atc agc tgg gtg cga cag gcc cct gga caa ggg ctt gag tgg atg gga tgg atc agc gct tac act ggt gac aca gac tat gca cag aaa ttc cag ggg cga gtc acc atg acc aca gat aca tcc acg aac aca gcc tac atg gaa ctg agg agc ctg aga tct gac gac gcg gcc gta tat tac tgt gcg aga ctt cgt ttg cag ggt gaa gtg gtg gtc cct cct agt caa tcc aat tgg ttc gac ccc tgg ggc cag gga acc ctg gtc acc gtc tcc tca
16	AT10_004	Light chain	gaa att gtg ttg aca cag tct cca gcc acc ctg tct ttg tat cca ggg gaa aga gcc acc ctc tct tgc agg gcc agt cag agt gtt agc agg tac tta gcc tgg tac caa cag aaa cct ggc cag gct ccc agg ctc ctc atc tat gat gca tcc aac agg gcc act ggc atc cca gcc agg ttc agt ggc agt ggg tct ggg aca gac ttc acc ctc acc atc agc agc cta gag cct gaa gat ttt gca gtt tat tac tgt cag cag cgt gac aac tgg ctt aag atc acc ttc ggc caa ggg aca cga ctg gaa att aaa gga act gtg
17	AT10_004 mutant A	Heavy chain CDR1	RH**A**IS
18	AT10_004 mutant A	Heavy chain CDR2	WISAYTGDTDYAQKFQG
19	AT10_004 mutant A	Heavy chain CDR3	LRLQGEVVVPPSQSNWFDP
20	AT10_004 mutant A	Light chain CDR1	RASQSVSRYLA
21	AT10_004 mutant A	Light chain CDR2	DASNRAT
22	AT10_004 mutant A	Light chain CDR3	QQRSNWLK
23	AT10_004 mutant A	Heavy chain	QVQLVQSGAEVRKPGASVKVSCKASGYTFTRH**A**ISWVRQAPGQGLEWMGWISAYTGDTDY AQKFQGRVTMTTDTSTNTAYMELRSLRSDDAAVYYCARLRLQGEVVVPPSQSNWFDPWGQ GTLVTVSS
24	AT10_004 mutant A	Light chain	EIVLTQSPATLSLYPGERATLSCRASQSVSRYLAWYQQKPGQAPRLLIYDASNRATGIPARFS GSGSGTDFTLTISSLEPEDFAVYYCQQRSNWLKITFGQGTRLEIKGTV

(continued)

SEQ ID NO	Antibody	Identity	Sequence
25	AT10_004 mutant A	Heavy chain CDR1	agg cat g**c**t atc agc
26	AT10_004 mutant A	Heavy chain CDR2	tgg atc agc gct tac act ggt gac aca gac tat gca cag aaa ttc cag ggg
27	AT10_004 mutant A	Heavy chain CDR3	ctt cgt ttg cag ggt gaa gtg gtg gtc cct cct agt caa tcc aat tgg ttc gac ccc
28	AT10_004 mutant A	Light chain CDR1	agg gcc agt cag agt gtt agc agg tac tta gcc
29	AT10_004 mutant A	Light chain CDR2	gat gca tcc aac agg gcc act
30	AT10_004 mutant A	Light chain CDR3	cag cag cgt agc aac tgg ctt aag
31	AT10_004 mutant A	Heavy chain	cag gtt cag ctg gtg cag tct gga gct gag gtg agg aag cct ggg gcc tca gtg aag gtc tcc tgc aag gct tcc ggt tac acg ttt acc agg cat g**c**t atc agc tgg gtg cga cag gcc cct gga caa ggg ctt gag tgg atg gga tgg atc agc gct tac act ggt gac aca gac tat gca cag aaa ttc cag ggg cga gtc acc atg acc aca gat aca tcc acg aac aca gcc tac atg gaa ctg agg agc ctg aga tct gac gac gcg gcc gta tat tac tgt gcg aga ctt cgt ttg cag ggt gaa gtg gtg gtc cct cct agt caa tcc aat tgg ttc gac ccc tgg ggc cag gga acc ctg gtc acc gtc tcc tca
32	AT10_004 mutant A	Light chain	gaa att gtg ttg aca cag tct cca gcc acc ctg tct ttg tat cca ggg gaa aga gcc acc ctc tct tgc agg gcc agt cag agt gtt agc agg tac tta gcc tgg tac caa cag aaa cct ggc cag gct ccc agg ctc ctc atc tat gat gca tcc aac agg gcc act ggc atc cca gcc agg ttc agt ggc agt ggg tct ggg aca gac ttc acc ctc acc atc agc agc cta gag cct gaa gat ttt gca gtt tat tac tgt cag cag cgt agc aac tgg ctt aag atc acc ttc ggc caa ggg aca cga ctg gaa att aaa gga act gtg
33	AT10_004 mutant B	Heavy chain CDR1	RHGIS
34	AT10_004 mutant B	Heavy chain CDR2	WISAYTGDTDYAQKFQG
35	AT10_004 mutant B	Heavy chain CDR3	LRLQGEVVVPPSQSNWFDP
36	AT10_004 mutant B	Light chain CDR1	RASQSVSRYLA

(continued)

SEQ ID NO	Antibody	Identity	Sequence
37	AT10_004 mutant B	Light chain CDR2	DASNRAT
38	AT10_004 mutant B	Light chain CDR3	QQR**Y**NWLK
39	AT10_004 mutant B	Heavy chain	QVQLVQSGAEVRKPGASVKVSCKASGYTFTRHGISWVRQAPGQGLEWMGWISAYTGDTDY AQKFQGRVTMTTDTSTNTAYMELRSLRSDDAAVYYCARLRLQGEVVVPPSQSNWFDPWGQ GTLVTVSS
40	AT10_004 mutant B	Light chain	EIVLTQSPATLSLYPGERATLSCRASQSVSRYLAWYQQKPGQAPRLLIYDASNRATGIPARFS GSGSGTDFTLTISSLEPEDFAVYYCQQR**Y**NWLKITFGQGTRLEIKGTV
41	AT10_004 mutant B	Heavy chain CDR1	agg cat ggt atc agc
42	AT10_004 mutant B	Heavy chain CDR2	tgg atc agc gct tac act ggt gac aca gac tat gca cag aaa ttc cag ggg
43	AT10_004 mutant B	Heavy chain CDR3	ctt cgt ttg cag ggt gaa gtg gtg gtc cct cct agt caa tcc aat tgg ttc gac ccc
44	AT10_004 mutant B	Light chain CDR1	agg gcc agt cag agt gtt agc agg tac tta gcc
45	AT10_004 mutant B	Light chain CDR2	gat gca tcc aac agg gcc act
46	AT10_004 mutant B	Light chain CDR3	cag cag cgt **ta**c aac tgg ctt aag
47	AT10_004 mutant B	Heavy chain	cag gtt cag ctg gtg cag tct gga gct gag gtg agg aag cct ggg gcc tca gtg aag gtc tcc tgc aag gct tcc ggt tac acg ttt acc agg cat ggt atc agc tgg gtg cga cag gcc cct gga caa ggg ctt gag tgg atg gga tgg atc agc gct tac act ggt gac aca gac tat gca cag aaa ttc cag ggg cga gtc acc atg acc aca gat aca tcc acg aac aca gcc tac atg gaa ctg agg agc ctg aga tct gac gac gcg gcc gta tat tac tgt gcg aga ctt cgt ttg cag ggt gaa gtg gtg gtc cct cct agt caa tcc aat tgg ttc gac ccc tgg ggc cag gga acc ctg gtc acc gtc tcc tca

(continued)

SEQ ID NO	Antibody	Identity	Sequence
48	AT10_004 mutant B	Light chain	gaa att gtg ttg aca cag tct cca gcc acc ctg tct ttg tat cca ggg gaa aga gcc acc ctc tct tgc agg gcc agt cag agt gtt agc agg tac tta gcc tgg tac caa cag aaa cct ggc cag gct ccc agg ctc ctc atc tat gat gca tcc aac agg gcc act ggc atc cca gcc agg ttc agt ggc agt ggg tct ggg aca gac ttc acc ctc acc atc agc agc cta gag cct gaa gat ttt gca gtt tat tac tgt cag cag cgt **ta**c aac tgg ctt aag atc acc ttc ggc caa ggg aca cga ctg gaa att aaa gga act gtg
49	AT10_004 mutant C	Heavy chain CDR1	RHGIS
50	AT10_004 mutant C	Heavy chain CDR2	WISAYTGDTDYAQKFQG
51	AT10_004 mutant C	Heavy chain CDR3	LRLQGEVVVPPSQSNWFDP
52	AT10_004 mutant C	Light chain CDR1	RASQSVSRFLA
53	AT10_004 mutant C	Light chain CDR2	DASNRAT
54	AT10_004 mutant C	Light chain CDR3	QQR**Y**NWLK
55	AT10_004 mutant C	Heavy chain	QVQLVQSGAEVRKPGASVKVSCKASGYTFTRHGISWVRQAPGQGLEWMGWISAYTGDTDY AQKFQGRVTMTTDTSTNTAYMELRSLRSDDAAVYYCARLRLQGEVVVPPSQSNWFDPWGQ GTLVTVSS
56	AT10_004 mutant C	Light chain	EIVLTQSPATLSLYPGERATLSCRASQSVSR**F**LAWYQQKPGQAPRLLIYDASNRATGIPARFS GSGSGTDFTLTISSLEPEDFAVYYCQQR**Y**NWLKITFGQGTRLEIKGTV
57	AT10_004 mutant C	Heavy chain CDR1	agg cat ggt atc agc
58	AT10_004 mutant C	Heavy chain CDR2	tgg atc agc gct tac act ggt gac aca gac tat gca cag aaa ttc cag ggg
59	AT10_004 mutant C	Heavy chain CDR3	ctt cgt ttg cag ggt gaa gtg gtg gtc cct cct agt caa tcc aat tgg ttc gac ccc
60	AT10_004 mutant C	Light chain CDR1	agg gcc agt cag agt gtt agc agg t**t**c tta gcc

(continued)

SEQ ID NO	Antibody	Identity	Sequence
61	AT10_004 mutant C	Light chain CDR2	gat gca tcc aac agg gcc act
62	AT10_004 mutant C	Light chain CDR3	cag cag cgt tac aac tgg ctt aag
63	AT10_004 mutant C	Heavy chain	cag gtt cag ctg gtg cag tct gga gct gag gtg agg aag cct ggg gcc tca gtg aag gtc tcc tgc aag gct tcc ggt tac acg ttt acc agg cat ggt atc agc tgg gtg cga cag gcc cct gga caa ggg ctt gag tgg atg gga tgg atc agc gct tac act ggt gac aca gac tat gca cag aaa ttc cag ggg cga gtc acc atg acc aca gat aca tcc acg aac aca gcc tac atg gaa ctg agg agc ctg aga tct gac gac gcg gcc gta tat tac tgt gcg aga ctt cgt ttg cag ggt gaa gtg gtg gtc cct cct agt caa tcc aat tgg ttc gac ccc tgg ggc cag gga acc ctg gtc acc gtc tcc tca
64	AT10_004 mutant C	Light chain	gaa att gtg ttg aca cag tct cca gcc acc ctg tct ttg tat cca ggg gaa aga gcc acc ctc tct tgc agg gcc agt cag agt gtt agc agg ttc tta gcc tgg tac caa cag aaa cct ggc cag gct ccc agg ctc ctc atc tat gat gca tcc aac agg gcc act ggc atc cca gcc agg ttc agt ggc agt ggg tct ggg aca gac ttc acc ctc acc atc agc agc cta gag cct gaa gat ttt gca gtt tat tac tgt cag cag cgt tac aac tgg ctt aag atc acc ttc ggc caa ggg aca cga ctg gaa att aaa gga act gtg

Table 2. Binding constants for AT10-004 and mutants

Antibody:	k_a:	k_d:	K_D:
AT10-004	1.4 (±0.1)	0.1	70 (± 10)
AT10-004, mutant A	0	-	-
AT10-004, mutant B	1.9 (± 0.1)	0.1	50 (± 10)
AT10-004, mutant C	1.7 (± 0.1)	0.1	60 (± 10)
k_a in 10^4 $sec^{-1}*M^{-1}$, k_d in 10^{-5} sec^{-1}, K_D in pM Constants were fitted in Scrubber2, using a global fit to a 1:1 interaction model			

Claims

1. A method for producing high affinity antibodies specific for an antigen of interest comprising:

   a) selecting a B-cell capable of producing antibody specific for said antigen of interest or selecting a B-cell capable of differentiating into a B-cell which is capable of producing antibody specific for said antigen of interest;
   b) providing said B-cell with a nucleic acid molecule encoding BCL6;
   c) providing said B-cell with a nucleic acid molecule encoding Bcl-xL;
   d) providing said B-cell with IL21 and CD40L and allowing expansion of said B-cell into a population of said B-cells;
   e) selecting at least one B-cell from said population of B-cells producing a B-cell receptor with a binding affinity higher than the average binding affinity of said population of B-cells for said antigen of interest;
   f) allowing expansion of said B-cell into a population of said B-cells;
   g) selecting at least one B-cell from said population of B-cells that produces antibody with a binding affinity higher than the average binding affinity of the antibodies produced by said population of B-cells for said antigen of interest;
   h) culturing said at least one B-cell into a population of B-cells; and
   i) obtaining antibodies produced by the B-cell culture.

2. A method according to claim 1 wherein said at least one B-cell is cultured for at least four weeks.

3. A method according to any one of the preceding claims, wherein said B-cell selected in step a) is a memory B-cell.

4. A method according to any one of the preceding claims, wherein said B-cell selected in step a) is a human memory B-cell.

5. A method according to any one of the preceding claims further comprising providing said B-cell with a growth factor.

6. A method according to any one of the preceding claims, wherein said B-cell selected in step a) originates from an individual which had been previously exposed to said antigen of interest.

7. A method according to any one of the preceding claims further comprising expressing a gene derived of said at least one B cell encoding the Ig heavy chain and/or Ig light chain in a second cell.

Patentansprüche

1. Verfahren zur Herstellung hochaffiner Antikörper, spezifisch für ein Antigen von Interesse, umfassend:

   a) Auswählen einer B-Zelle, die geeignet ist, Antikörper spezifisch für das Antigen von Interesse herzustellen oder Auswählen einer B-Zelle, die geeignet ist, in eine B-Zelle zu differenzieren, die geeignet ist, Antikörper spezifisch für das Antigen von Interesse herzustellen;
   b) Versehen der B-Zelle mit einem Nukleinsäuremolekül, kodierend BCL6;
   c) Versehen der B-Zelle mit einem Nukleinsäuremolekül, kodierend Bcl-xL;
   d) Versehen der B-Zelle mit IL21 und CD40L und Erlauben einer Expansion der B-Zelle in eine Population der

B-Zellen;
e) Auswählen mindestens einer B-Zelle aus der Population von B-Zellen, herstellend einen B-Zellrezeptor mit einer Bindeaffinität höher als die durchschnittliche Bindeaffinität der Population von B-Zellen für das Antigen von Interesse;
f) Erlauben einer Expansion der B-Zelle in eine Population der B-Zellen;
g) Auswählen mindestens einer B-Zelle aus der Population von B-Zellen, die Antikörper herstellt mit einer Bindeaffinität höher als die durchschnittliche Bindeaffinität der Antikörper, hergestellt von der Population von B-Zellen für das Antigen von Interesse;
h) Kultivieren der mindestens einen B-Zelle zu einer Population von B-Zellen; und
i) Erhalten von Antikörpern, hergestellt von der B-Zellkultur.

2. Verfahren nach Anspruch 1, wobei die mindestens eine B-Zelle für mindestens vier Wochen kultiviert wird.

3. Verfahren nach einem der vorhergehenden Ansprüche, wobei die in Schritt a) ausgewählte B-Zelle eine Gedächtnis-B-Zelle ist.

4. Verfahren nach einem der vorhergehenden Ansprüche, wobei die in Schritt a) ausgewählte B-Zelle eine humane Gedächtnis-B-Zelle ist.

5. Verfahren nach einem der vorhergehenden Ansprüche, ferner umfassend Versehen der B-Zelle mit einem Wachstumsfaktor.

6. Verfahren nach einem der vorhergehenden Ansprüche, wobei die in Schritt a) ausgewählte B-Zelle von einem Individuum stammt, das zuvor dem Antigen von Interesse ausgesetzt war.

7. Verfahren nach einem der vorhergehenden Ansprüche, ferner umfassend Exprimieren eines Gens, abgeleitet von der mindestens einen B-Zelle, kodierend die Ig-Schwerkette und/oder Ig-Leichtkette in einer zweiten Zelle.

Revendications

1. Un procédé pour la production d'anticorps à affinité élevée spécifiques d'un antigène d'intérêt, comprenant:

a) sélectionner un lymphocyte B capable de produire un anticorps spécifique pour ledit antigène d'intérêt, ou sélectionner un lymphocyte B capable de différenciation en un lymphocyte B qui est capable de produire un anticorps spécifique pour ledit antigène d'intérêt;
b) fournir audit lymphocyte B une molécule d'acide nucléique codant pour BCL6;
c) fournir audit lymphocyte B une molécule d'acide nucléique codant pour Bcl-xL;
d) fournir IL21 et CD40L audit lymphocyte B puis permettre l'expansion dudit lymphocyte B dans une population desdits lymphocytes B;
e) sélectionner au moins un lymphocyte B à partir de ladite population de lymphocytes B produisant un récepteur de lymphocytes B présentant une affinité de liaison supérieure à l'affinité de liaison moyenne de ladite population de lymphocytes B pour ledit antigène d'intérêt;
f) permettre l'expansion dudit lymphocyte B dans une population desdits lymphocytes B;
g) sélectionner au moins un lymphocyte B à partir de ladite population de lymphocytes B qui produit l'anticorps présentant une affinité de liaison supérieure à l'affinité de liaison moyenne des anticorps produits par ladite population de lymphocytes B pour ledit antigène d'intérêt;
h) la culture dudit au moins un des lymphocytes B dans une population de lymphocytes B; et
i) l'obtention d'anticorps produits par la culture de lymphocytes B.

2. Un procédé selon la revendication 1, dans lequel ledit au moins un lymphocyte B est mis en culture pendant au moins quatre semaines.

3. Un procédé selon l'une quelconque des revendications précédentes, dans lequel ledit lymphocyte B sélectionné dans l'étape a) est un lymphocyte B de mémoire.

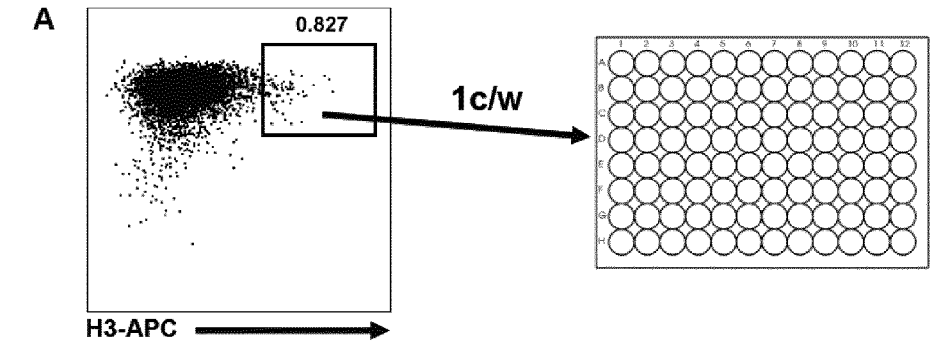
4. Un procédé selon l'une quelconque des revendications précédentes, dans lequel ledit lymphocyte B sélectionné dans l'étape a) est un lymphocyte B de mémoire humaine.

5. Un procédé selon l'une quelconque des revendications précédentes, comprenant en outre la fourniture audit lymphocyte B d'un facteur de croissance.

6. Un procédé selon l'une quelconque des revendications précédentes, dans lequel ledit lymphocyte B sélectionné dans l'étape a) provient d'un individu qui a été préalablement exposé audit antigène d'intérêt.

7. Un procédé selon l'une quelconque des revendications précédentes, comprenant en outre l'expression d'un gène dérivé dudit au moins un lymphocyte B codant pour la chaîne lourde d'Ig et/ou la chaîne légère d'Ig d'un deuxième lymphocyte.

Figure 1



B

H3 ELISA

0.05	0.06	0.05	0.05
0.78	0.05	0.56	0.05
0.05	0.75	0.59	0.04
0.73	0.70	0.04	0.04

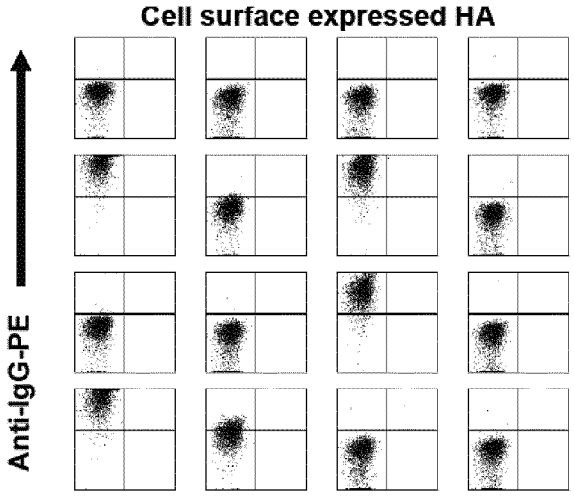


Figure 2

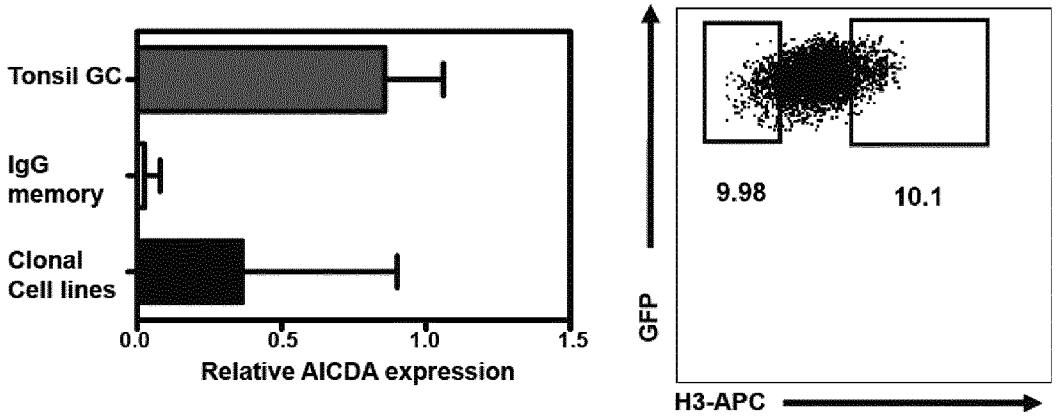
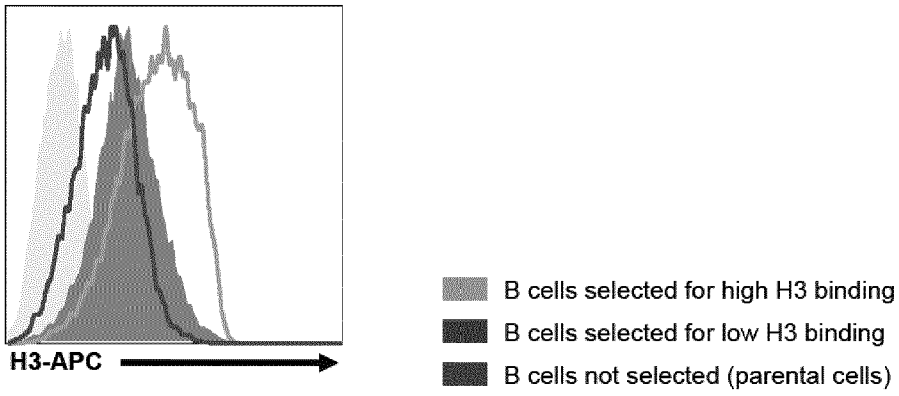

Tonsil GC
IgG memory
Clonal Cell lines
0.0
0.5
1.0
1.5
Relative AICDA expression
9.98
10.1
GFP
H3-APC

Figure 3

A


H3-APC
B cells selected for high H3 binding
B cells selected for low H3 binding
B cells not selected (parental cells)

B

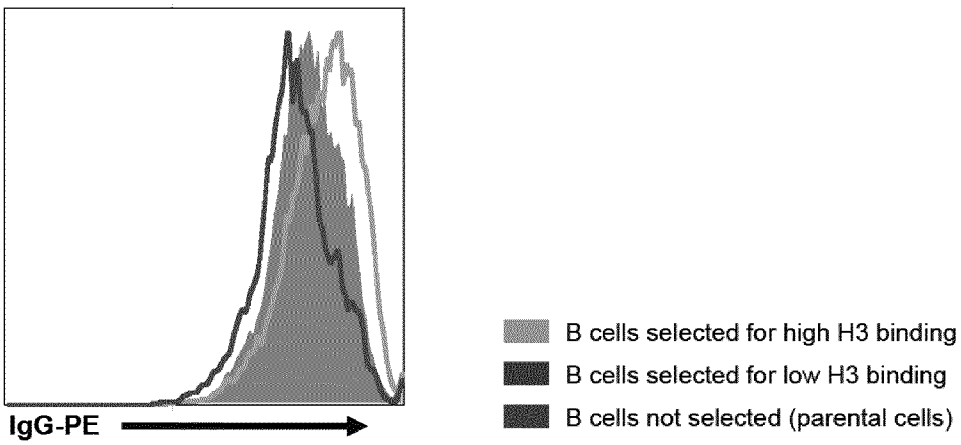

IgG-PE
B cells selected for high H3 binding
B cells selected for low H3 binding
B cells not selected (parental cells)

Figure 4

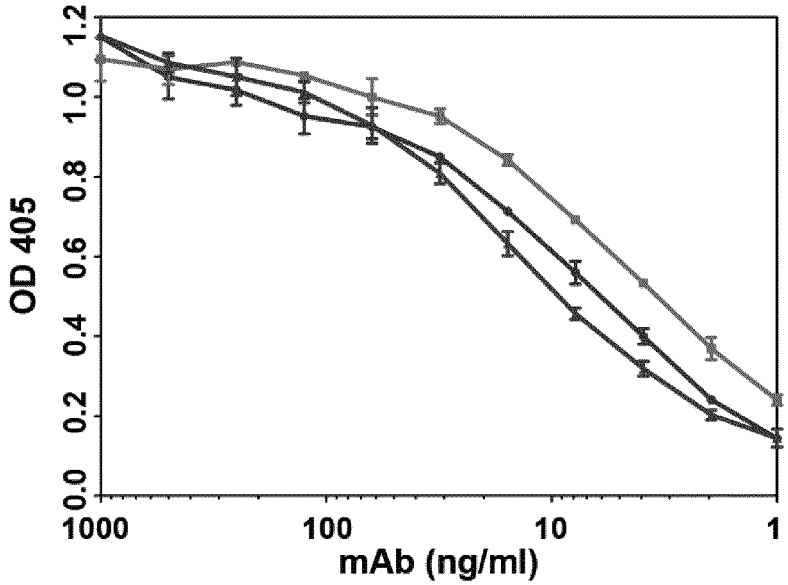


Figure 5

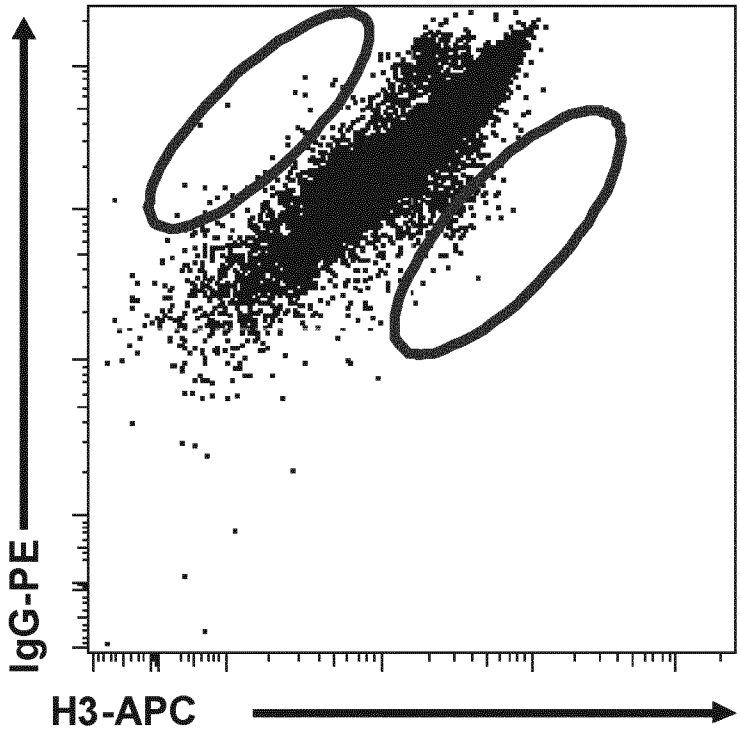


Figure 6

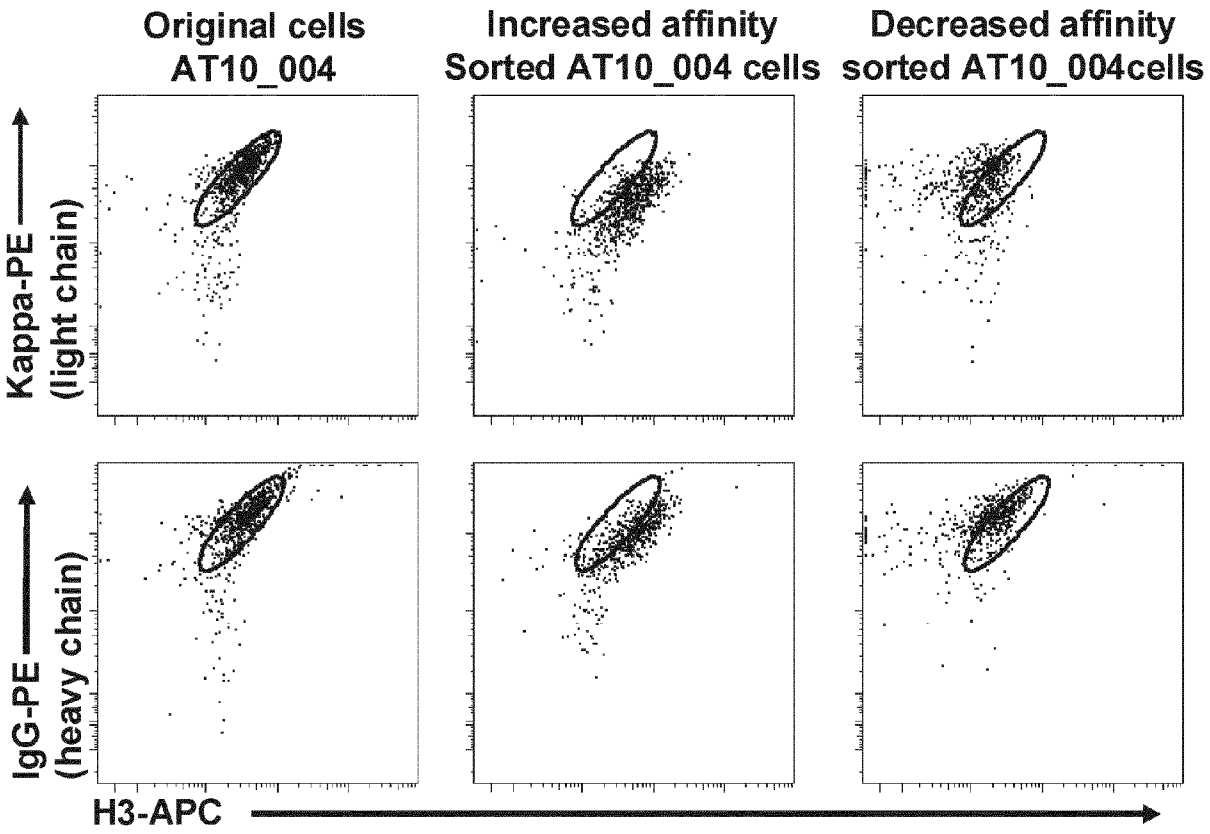

Original cells
AT10_004
Increased affinity
Sorted AT10_004 cells
Decreased affinity
sorted AT10_004cells
Kappa-PE
(light chain)
IgG-PE
(heavy chain)
H3-APC

Figure 7

		Kappa light chain, CDR3 Position 108	Kappa light chain, CDR1 Position 38	Heavy chain, CDR1 Position 38
Parental cells	AT10_004	S	Y	G
Decreased affinity cells	AT10_004 mutant A	S	Y	A
Increased affinity cells	AT10_004 mutant B	Y	Y	G
Increased affinity cells	AT10_004 mutant C	Y	F	G

Figure 8

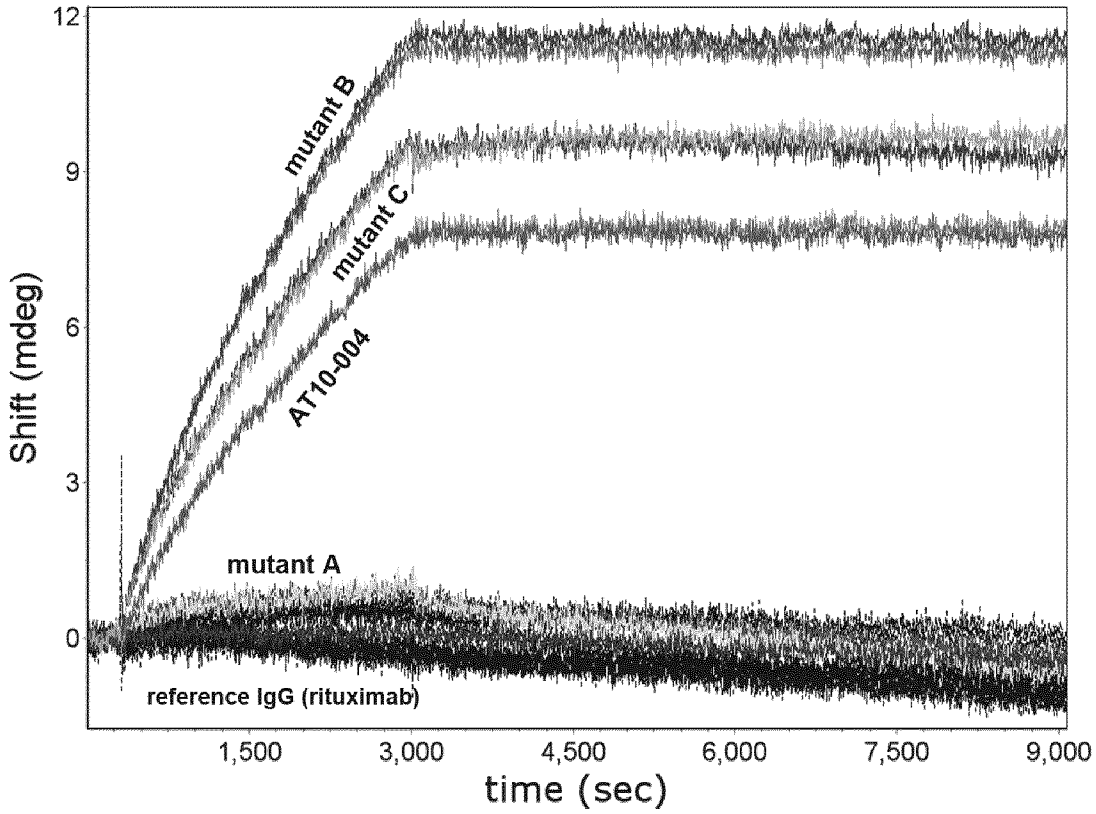
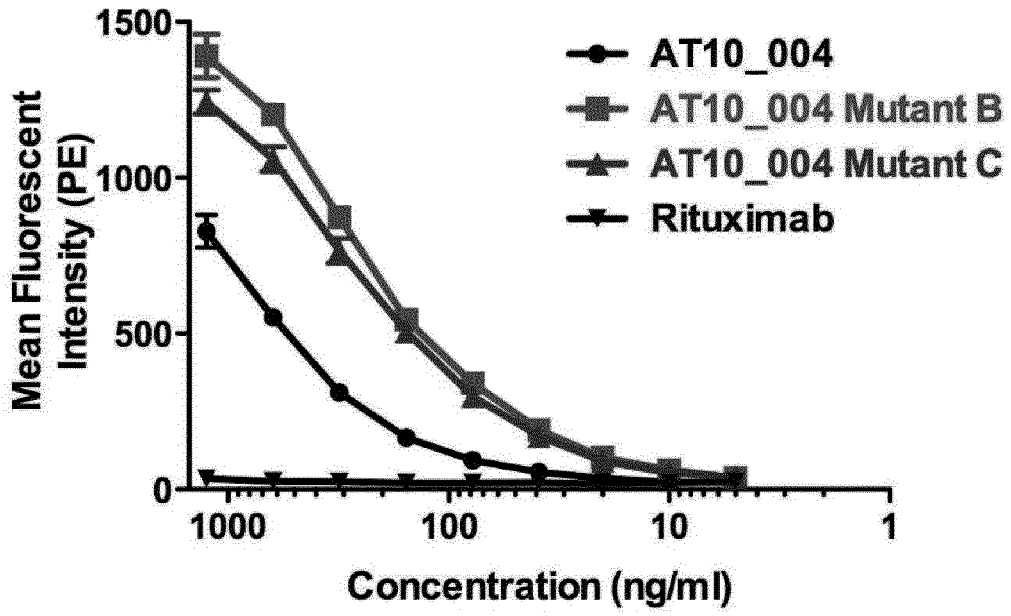

12
9
6
3
0
Shift (mdeg)
mutant B
mutant C
AT10-004
mutant A
reference IgG (rituximab)
1,500
3,000
4,500
6,000
7,500
9,000
time (sec)

Figure 9


1500
1000
500
0
Mean Fluorescent Intensity (PE)
1000
100
10
1
Concentration (ng/ml)
AT10_004
AT10_004 Mutant B
AT10_004 Mutant C
Rituximab

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- WO 2007067046 A **[0005] [0008] [0014] [0016] [0047]**
- US 2008305076 A **[0021]**

Non-patent literature cited in the description

- **SEAN A DIEHL ; HEIKE SCHMIDLIN ; MAHO NAGASAWA ; SIMON D VAN HAREN ; MARK J KWAKKENBOS ; ETSUKO YASUDA ; TIM BEAUMONT ; FERENC A SCHEEREN.** Hergen Spits STAT3-mediated up-regulation of BLIMP1 is coordinated with BCL6 down-regulation to control human plasma cell differentiation. *J Immunol,* 2008, vol. 180 (7), 4805-15 **[0007]**
- **KWAKKENBOS et al.** Generation of stable monoclonal antibody-producing B cell receptor- positive human memory B cells by genetic programming. *Nature Medicine,* vol. 16 (1), 123-8 **[0047]**
- **KWAKKENBOS et al.** Figure 2 left panel and 'Generation of stable monoclonal antibody-producing B cell receptor-positive human memory B cells by genetic programming. *Nature Medicine,* 2010, vol. 16 (1), 123-8 **[0048]**
- **LOKATE et al.** *J. Am. Chem. Soc.,* 2007, vol. 129, 14013-140318 **[0054]**

ESZKÖZÖK ÉS ELJÁRÁSOK NAGY AFFINITÁSÚ ANTITESTEK ELŐÁLLÍTÁSÁRA

Szabadalmi igénypontok

1. Eljárás nagy affinitású antitestek előállítására, amelyek specifikusak egy releváns (kívánt) antigénre (angolul: „antigen of interest"), ahol az magában foglalja a következőket:
   a) egy B-sejt kiválasztása, amely képes olyan antitest előállítására, amely specifikus a nevezett releváns (kívánt) antigénre, vagy egy B-sejt kiválasztása, amely képes differenciálódni egy B-sejtté, amely képes olyan antitest előállítására, amely specifikus a nevezett releváns (kívánt) antigénre;
   b) a nevezett B-sejt ellátása egy nukleinsav molekulával, amely kódolja a BCL6-ot;
   c) a nevezett B-sejt ellátása egy nukleinsav molekulával, amely kódolja a Bcl-xL-t;
   d) a nevezett B-sejt ellátása IL21-gyel és CD40L-lel és a nevezett B-sejt expanziójának a lehetővé tétele a nevezett B-sejtek egy populációjába;
   e) legalább egy B-sejt kiválasztása a B-sejtek nevezett populációjából, amely által egy B-sejt receptor van előállítva, amely egy olyan kötődési affinitással rendelkezik, amely nagyobb, mint a B-sejtek nevezett populációjának az átlagos kötődési affinitása a nevezett releváns (kívánt) antigén iránt;
   f) a nevezett B-sejt expanziójának a lehetővé tétele a nevezett B-sejtek egy populációjába;
   g) legalább egy B-sejt kiválasztása a B-sejtek nevezett populációjából, amely által olyan antitest van előállítva, amely egy olyan kötődési affinitással rendelkezik, amely nagyobb, mint a B-sejtek nevezett populációja által előállított antitesteknek az átlagos kötődési affinitása a nevezett releváns (kívánt) antigén iránt;
   h) a nevezett legalább egy B-sejt tenyésztése, hogy B-sejtek egy populációja legyen létrehozva; és
   i) a B-sejt-kultúra (B-sejt-tenyészet) által előállított antitestek kinyerése.
2. Eljárás az 1. igénypont szerint, ahol a nevezett legalább egy B-sejt legalább négy hétig van tenyésztve.
3. Eljárás az előző igénypontok bármelyike szerint, ahol a nevezett B-sejt, amely az a) lépésben van kiválasztva, egy memória B-sejt.
4. Eljárás az előző igénypontok bármelyike szerint, ahol a nevezett B-sejt, amely az a) lépésben van kiválasztva, egy humán memória B-sejt.
5. Eljárás az előző igénypontok bármelyike szerint, amely továbbá magában foglalja a nevezett B-sejt ellátását egy növekedési faktorral.
6. Eljárás az előző igénypontok bármelyike szerint, ahol a nevezett B-sejt, amely az a) lépésben van kiválasztva, egy olyan egyéntől származik, amely azelőtt a nevezett releváns (kívánt) antigén hatásának volt kitéve.
7. Eljárás az előző igénypontok bármelyike szerint, amely továbbá magában foglalja egy gén expresszálását, amely a nevezett legalább egy B-sejtből származik, ahol az kódolja az Ig-nehézláncot és/vagy Ig-könnyűláncot egy második sejtben.